Molecular physiology of weak organic acid stress in Bacillus subtilis
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CHAPTER 7

GENERAL DISCUSSION
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Many studies on the effectiveness of weak organic acid (WOA) food preservatives have been published, however details on their differential modes of action are limited. More detailed knowledge of the mode of action of WOAs may allow more effective application and help discover more effective preservatives. Also, it is crucial to understand which cells are most resistant to WOA stress, as these will ultimately continue to grow and spoil food. In this thesis more detailed knowledge on the effects that sorbic and acetic acid have on Bacillus subtilis is described.

Weak organic acid stress affects cells in several ways: The cytosolic pH is decreased and some weak acids also deplete the membrane potential. As the cell membrane forms the defining border of the cell, this is also its primary line of defence. Corroborating its importance in cellular defence, membrane alterations were observed upon exposure of B. subtilis to weak organic acid stress. These effects of WOA stress are discussed below in more detail.

INTRACELLULAR pH

As the most distinctive feature of WOA stress was presumed to be an induced rapid decrease of cytosolic pH, we first described a method to measure the pH inside the cell on-line. We confirmed our hypothesis by showing the dynamics of the internal pH values of B. subtilis during batch growth in the absence and presence of WOA stress. We then set out to describe how weak organic acid preservatives work. The decrease of the pH, is proportional to the concentration of added acid, although this has only been tested in this thesis for sorbic and acetic acid. Both acids which have a similar pKa value. The internal pH appears to have a direct effect on the rate of glucose consumption and likely as well on many other enzymatic reactions. To comprehensively describe the level of acidification by WOA preservatives, the level of intracellular accumulated anion also needs to be determined. This can be investigated with acids for which radioactive isotopes are readily available. Together with the level of anion accumulation in both energized and starved cells, the cytoplasmic buffer capacity needs to be determined to understand how many protons are released inside the cell upon WOA stress and if there is adaptation to WOA stress induced cytoplasmatic acidification.

Different microbial species may have different buffering capacities and alterations of this buffer capacity may also constitute a coping mechanism for WOA stress. In chapter 3, we have assumed that the intracellular concentration of the anion could be estimated using the ΔpH and extracellular anion concentration. This does not need to be true in energized cells, in which protons are pumped out and the anion may diffuse over the membrane or be actively extruded. With the buffer capacity and actual anion accumulation established, it can be decided if the Henderson – Hasselbalch equation may be applied to energized
cells to calculate the intracellular concentration of weak acid anion. If so, the \( \text{pH}_i \) calculations as described in chapter 3 can likely be applied.

**Membrane potential**

Only limited quantitative data has been published on the effects weak organic acid preservatives have on the membrane potential. Regarding sorbic acid, several measurements have been published that hint to a role as an uncoupler, but quantitative data was not given in these papers. In chapter 3 we have shown that sorbic acid can indeed act as a protonophore. This means that the anion is likely capable of traversing the membrane at a significant rate.

The effect that sorbate has on the membrane potential is much less apparent for acetic acid. Our results suggest that a decrease in growth rate evoked by a lowered \( \Delta \psi \) will mainly affect cell division. This might explain the WOA stress hypersensitive phenotype observed in the *B. subtilis rodZ::Tn10* mutant. The observed phenotype of this mutant strongly resembles the morphology of *B. subtilis* cells with a disrupted cytoskeleton. As such a disruption may be due to a depletion of the membrane potential, this may explain the extreme sensitivity of the *rodZ* mutant. In this mutant, MreB (which can bind to RodZ) is always delocalized. Upon depletion of \( \Delta \psi \), this delocalizing effect may be even more pronounced, thus further inhibiting cell growth.

A quantitative description of the influence of \( \Delta \psi \) depletion on growth needs to be established for WOA preservatives. The \( \Delta \psi \) depletion by various WOA preservatives should be considered a prime parameter in future experiments to elucidate their mode of action. To this end, it would be highly beneficial if a \( \Delta \psi \) probe were to be developed that is as efficient and versatile as pHluorin is for \( \text{pH}_i \) measurements. For such a probe, similar criteria as set in chapters 2 and 6 can be applied e.g. the probe used to measure \( \Delta \psi \) should maintain accuracy over the \( \Delta \psi \) range assessed (-200 – 0 mV) and both the presence of the probe itself in a cell as well as the detection method applied should have minimal effect on cell physiology. Apart from using specific dyes, at this moment, three likely candidates may be developed into genetically encoded probes for \( \Delta \psi \). A GFP bound to a protein that delocalizes with depleting \( \Delta \psi \) (28) might be envisaged, but my unpublished results suggest that a GFP fused with the membrane binding helix of MinD may interfere with cell division. Also, in general, this approach would be difficult to quantify (possibly only by microscope) and influenced by the membrane composition. Another option would be trying to adapt a proteorhodopsin (PROPS, (225)) for ratiometric measurements. It has an excellent sensitivity range and change in fluorescence intensity change over the above indicated \( \Delta \psi \) range. The downside of using a proteorhodopsin lies mainly in the low fluorescence intensity, approximately 1/1000 of GFP. Also, it requires a retinal as a cofactor, which should either be added externally or synthesized by the cell itself. The third option would be fusing a fluorescent reporter to the sensor domain of a
voltage sensitive protein (such as voltage-gated K⁺ channels (e.g. Shaker (226, 227)). These have been used quite extensively in neurobiology to monitor action potentials and were therefore aimed at monitoring a more positive membrane potential, also the change in fluorescence intensity in that voltage domain appears to be limited. However, combined with the latest fluorescent proteins optimized for quantum yield (228) these may be the most adaptable and easiest to implement in bacterial electrophysiology.cell as well as the detection method applied should have minimal effect on cell physiology.

**Membrane composition**

When exposed to sorbic acid, *B. subtilis* modifies its membrane composition. The membrane composition of the *B. subtilis rodZ::Tn10 mutant was also different from the wild-type. Both in the lipid fraction of the mutant cells as well as in that of the cells exposed to sorbic acid stress we observed an increased acyl chain length in conjunction with increased branching of such chains. Also, cardiolipin levels were decreased in the mutant but increased in the WT strain upon sorbic acid stress. These two observations suggest that the acyl chain adaptation of the mutant to lowered RodZ, and PgsA, activity had approached a limit that could not be stretched further to effectively adapt to induced sorbic acid stress.

The rate of diffusion of weak organic acids over the cell membrane of *B. subtilis* is higher than can be assessed by the methods employed in chapters 3 and 5. It is therefore not possible to draw a definitive conclusion about a role of membrane permeability and neutral acid diffusion. However, the initial (neutral) acid influx rate plays only a minor role in the decrease in growth rate. Initially, the pH recovers rapidly and the rate limiting step with uncouplers is the re-entry of the anion to face the interior leaflet of the plasmamembrane. It is therefore more relevant to determine the rate of acidification due to anion shuttling. Here, an altered membrane composition may play a role (as is likely the case with the efficient uncoupler CCCP), although from literature and the data present in this thesis, this is not immediately apparent or fully understood. In the data presented here, shuttling of the (sorbate) anion causes a depletion of the membrane potential. This has not yet been tested with “adapted” cells, nor has a rate of diffusion been determined. To proof that the altered membrane composition results in a change in shuttling rate, membrane conductivity measurements will have to be performed (229, 230)

The permeability to either weak organic acid preservatives or proton remains an important parameter for the cell, as it is known to change with temperature. To this end, reconstituted membrane vesicles or lipid bilayers may be examined, using lipids from weak acid-adapted and non-adapted cells.

**Metabolism**

Fermentation acids are commonly used as preservatives. These have the particular effect that they may shift the equilibrium of metabolic reactions, which will interfere with
regular metabolism. Other weak acid preservatives do not necessarily affect metabolic routes. While interference with metabolic routes may be advantageous, fermentation acids may also more readily be consumed/neutralized by microorganisms.

**PERSPECTIVES**

Other weak acid preservatives should be tested for their ability to deplete Δψ and if their effect on pHi agrees with the findings described in chapter 3. When a quantitative description of the relation between Δψ and growth rate is available, and the effects on pHi known, the specific effects of weak organic acids can be separated and assigned more accurately. This may allow more accurate predictions of bacterial growth with these preservatives as well allow appropriate combinations of preservation strategies to optimize their (synergistic) effectiveness.

In the near future, single cell pHi measurements may shed light on growth rate distribution within a small population of cells. Also, pHi recovery of individual cells may be seen. Such data will help predictive modelling of bacterial growth stressed with weak organic acids and contribute to improved microbial food stability and food safety (231).